Foxp3 interacts with nuclear factor of activated T cells and NF-κB to repress cytokine gene expression and effector functions of T helper cells

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Scurfy mice, which are deficient in a functional Foxp3, exhibit a severe lymphoproliferative disorder and display generalized overproduction of cytokines. Here, we show that, among the Foxp transcriptional factor family, which includes Foxp1, Foxp2, and Foxp3, only Foxp3 has the ability to inhibit IL-2, IL-4, and IFN-γ production by primary T helper cells. We found that Foxp3 physically associates with the Rel family transcription factors, nuclear factor of activated T cells (NFAT) and NF-κB, and blocks their ability to induce the endogenous expression of their target genes, including key cytokine genes. More importantly, T cells derived from scurfy mice have a dramatic increase in nuclear factor of activated T cells (NFAT) and NF-κB transcriptional activity compared with the T cells derived from WT mice. Furthermore, complementation of Foxp3 in scurfy-derived T cells lowers the NFAT and NF-κB transcriptional activity to the physiological level. Finally, we show that myelin proteolipid protein-specific autoreactive T cells transduced with Foxp3 cannot mediate experimental autoimmune encephalomyelitis, providing further support that Foxp3 suppresses the effector function of autoreactive T cells. Foxp3 has already been associated with the generation of CD4$^+$CD25$^+$ regulatory T cells; our data additionally demonstrate that Foxp3 suppresses the effector functions of T helper cells by directly inhibiting the activity of two key transcription factors, NFAT and NF-κB, which are essential for cytokine gene expression and T cell functions.

experimental autoimmune encephalomyelitis | transcription factors | CD4$^+$ T cells | interleukins

Fox proteins are identified by a conserved 110-aa DNA-binding domain (winged helix domain) (1–3). Among the Fox transcription factors, recent attention has been drawn to the members of the Foxp subfamily (Foxp1, Foxp2, and Foxp3). Foxp1 has been implicated as a tumor suppressor gene due to its loss in several types of tumors (including breast, lung, and stomach) and leukemia (4, 5). Interestingly, Foxp2 has been implicated in a specific familial language disorder, suggesting a role in neural development (6, 7). In humans, Foxp3 mutations cause X-linked autoimmunity-allergic disregulation syndrome, and the mouse Foxp3 mutation is responsible for the scurfy mouse phenotype (8–10). Initial studies of scurfy mice indicated that CD4$^+$ T cells from these mice were hyperresponsive to T cell antigen receptor (TCR) stimulation and produced excessive amounts of a number of cytokines. Adoptive transfer of scurfy-derived CD4$^+$ T cells into severe combined immunodeficient (SCID) recipients induced a rapid wasting disease (11–14). More recently, it has been shown that Foxp3 expression is highly enriched in CD4$^+$CD25$^+$ regulatory T cells (Tregs). Furthermore, forced expression of Foxp3 can convert murine naive T cells into regulatory T cells such that the cells phenotypically and functionally resemble naturally arising CD4$^+$CD25$^+$ Tregs. Furthermore, transfer of CD4$^+$CD25$^+$ T cells can delay the progression of autoimmune disease in Foxp3-deficient mice (15–17). Although the functional association of Foxp3 with Tregs provides a significant step toward our understanding of the generation of these regulatory cells, little is known about the biochemical mechanisms by which Foxp3 inhibits cytokine production in T cells. Although predicted to be a transcriptional repressor, no consensus DNA-binding sequence or protein partners have been identified in its sequence to predict the mechanism by which Foxp3 may mediate its inhibitory functions in T cells (18, 19).

In this study, we provide evidence that Foxp3, but not Foxp1 or Foxp2, is able to repress cytokine gene expression in primary CD4$^+$ T cells. Forced expression of Foxp3 dramatically suppresses endogenous cytokine expression driven by NFAT and NF-κB. Foxp3 physically associates with the REL domain proteins NFAT and NF-κB and suppresses their transcriptional activity. More importantly, Foxp3-deficient T cells, derived from scurfy mice, have a dramatic increase in NFAT and NF-κB transcriptional activity compared with the WT T cells. Complementation of Foxp3 into these Foxp3-deficient T cells reduces NFAT and NF-κB transcriptional activity closer to activity found at physiological levels. Proteolipid protein (PLP)-specific autoreactive T cells transduced by Foxp3 were incapable of mediating experimental autoimmune encephalomyelitis (EAE), providing further support that Foxp3 suppresses the effector function of autoreactive T cells. These experiments demonstrate that Foxp3 not only promotes the generation of T regulatory cells, but also inhibits cytokine production and T cell effector function. It achieves this additional role by repressing the activity of two key transcription factors, NFAT and NF-κB, which are essential for cytokine gene expression.

Materials and Methods

Mice. Mice were housed in a specific pathogen-free environment, and studies were conducted according to Public Health Service guidelines and with the approval of an Animal Care and Use Committee. The scurfy mice were obtained from The Jackson Laboratory. The 5B6 B10.S TCR transgenic mice specific for PLP139-151 have been described (20).

Reagents and Antibodies. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Calbiochem. Anti-Myc, anti-hemagglutinin (HA), and anti-P65 (C20) antibodies were obtained from Santa Cruz Biotechnology.

Transfection and Luciferase Assays. Transfection of 293 and Jurkat cells was performed as described (21). For primary T cells, 1.5×10^7 purified CD4$^+$ T cells were allowed to rest for 24 h in the presence of IL-2. They were then electroporated with 20 μg of

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Abbreviations: TCR, T cell antigen receptor; Treg, regulatory T cell; PLP, proteolipid protein; EAE, experimental autoimmune encephalomyelitis; HA, hemagglutinin; NFAT, nuclear factor of activated T cells; NFATp, NFAT, preexisting; NFAT-CA, constitutively active version of NFATp.

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NF-κB-Luc, NFAT-Luc, or 5xGal4-Luc, 20 μg of Gal4-P65 (350–1650), Gal4-NFAT (1–1450), Gal4-ELK1, or Gal4-KRC (21), and 15 μg of Foxp3 or pcDNA3 control as indicated, as well as with 2 μg of Tk-*Renilla*. The electroporated cells were stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 18 h and analyzed by means of the dual luciferase assay with relative normalization for *Renilla*.

EMSA and Western Blot Analysis. Effectene-mediated transfections of 293T cells were performed. To prepare cell extracts, cells were washed twice with PBS and lysed for 10 min on ice in 1 ml of Triton lysis buffer (25 mM Hepes, pH 7.5/250 mM NaCl/1% Triton X-100/10% glycerol/5 mM EDTA/1 mM DTT) and complete protease inhibitor mixture (Roche Molecular Biochemicals) The lysates were cleared by centrifugation for 10 min at 16,320 × *g*. The cell lysates were precleared with 30 μl of protein A/G-Sepharose beads and then incubated overnight with 25 μl of anti-Myc antibody directly conjugated to Sepharose beads. The immunoprecipitates were then washed five times with the lysis buffer, resuspended in SDS sample buffer, and heated at 95°C for 5 min. Immunoprecipitated proteins were separated by SDS/PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane. Western blotting was performed by probing with primary antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biosciences). EMSA was performed with nuclear extracts prepared from transfected 293T cells and the ^{32}P-labeled NF-κB oligonucleotides (Santa Cruz Biotechnology).

Retroviral Infection. MSCV GFP-RV retroviral DNA plasmids were transfected into the FNX-E packaging cell line by using Effectene (Qiagen, Valencia, CA). Retrovirus-containing supernatant was collected after 72 h of incubation with the transfected packaging cells. Freshly isolated, magnetic-activated cell sorting (MACS)-purified, CD4$^+$ T cells were activated by using plate-bound anti-CD3 and anti-CD28 or 30 μg/ml PLP peptide and antigen-presenting cells (APCs) when 5B6-derived CD4$^+$ T cells were used. After 24 h of activation, CD4$^+$ T cells were infected by resuspending cells in retrovirus-containing supernatant supplemented with 8 μg/ml Polybrene and recombinant human IL-2 (25 units/ml), followed by centrifugation for 50 min at 350 × *g*. After 48 h of incubation at 37°C, the GFP$^+$ cells were purified by FACS sorting and expanded for an additional 72 h in the presence of IL-2.

Adoptive Transfer Experiments and EAE Evaluation. CD4$^+$ T cells from PLP-specific TCR transgenic mice were purified, activated with 30 μg/ml PLP peptide 139–151, and infected with RV or RV-Foxp3 retroviruses. GFP$^+$ cells were sorted and injected into RAG2-deficient mice. The progression of EAE was scored daily as described (22)and is presented as the mean clinical disease among each group.

Expression Analysis by Real-Time PCR. RNA was prepared 24 and 48 h after transfection with TRIzol (Invitrogen), first-strand cDNA synthesis using oligo(dT) primers, and the iScript cDNA Synthesis Kit (Bio-Rad). Samples were subjected to real-time PCR analysis on an Applied Biosystems PRISM 7000 Sequencer Detection System (Applied Biosystems) under standard conditions. A20 primers were as follows: A20R, 5′-GCAGTTGGGCGTTTCACATT-3′; A20F, 5′-GCCCAGAATCCCATGGAAC-3′; A20 Probe (FAM)-CGTGCCCCAGCTTTCTCTCATGGAT-(TAMRA). Relative mRNA abundance was normalized against GAPDH (Applied Biosystems).

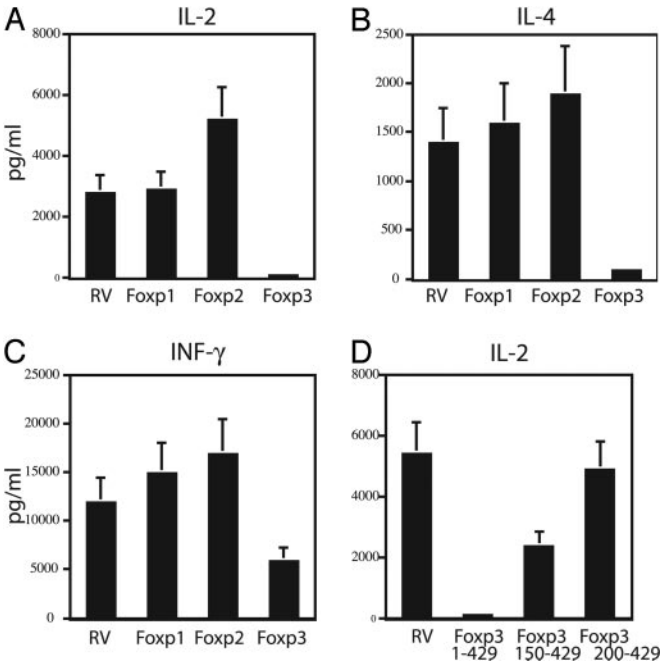

Fig. 1. Foxp3, but not other Foxp family members, suppresses cytokine expression in T cells. Foxp1, Foxp2, Foxp3, or deletion mutants of Foxp3 cDNAs (encoding the indicated amino acids) were inserted into the RV GFP retrovirus vector. These vectors simultaneously express a specific Foxp cDNA with the GFP, with the use of an internal ribosomal entry site (IRES). Purified T cells were stimulated with anti-CD3 and anti-CD28, and infected with the Foxp-expressing or control retroviruses in the presence of human IL-2. The GFP$^+$ T cells were sorted 48 h postinfection. One week after the infection, GFP$^+$-infected CD4$^+$ T cells were stimulated with antigen-presenting cells (irradiated splenocytes) plus soluble anti-CD3 antibody. The figure represents the amount of IL-2 (*A* and *D*), IL-4 (*B*), and IFN-γ (*C*) secreted in the culture supernatant of these activated cells, as measured by ELISA.

Results

Foxp3, but Not Foxp1 or Foxp2, Is Able to Turn Off Cytokine Expression in Primary CD4$^+$ T Cells. It has been shown *in vitro* that, although Foxp1, Foxp2, and Foxp3 all bind the Fox-binding site within the IL-2 promoter, only Foxp1 and Foxp3 are able to suppress IL-2 promoter activity (19, 23). First, we determined whether forced expression of the Foxp proteins in primary naive T cells could modulate cytokine expression. Biscistronic retroviral vectors expressing each of the Foxp genes and the GFP, or the GFP alone, were generated. Peripheral CD4$^+$ T cells were infected with Foxp-expressing retrovirus or the control retrovirus. GFP+ T cells were sorted and stimulated with anti-CD3 in the presence of antigen-presenting cells, and their cytokine production was then examined (Fig. 1). Interestingly only Foxp3, but not Foxp1 or Foxp2, expression in T cells suppressed the production of IL-2, IL-4, and IFN-γ (Fig. 1 *A–C*). We next generated deletion mutants of Foxp3 to further examine the specific parts of Foxp3 responsible for suppressing IL-2 production. The deletion of the first 150 aa of the Foxp3 protein diminished its ability to repress IL-2 expression. An extended deletion, which includes the zinc finger domain, completely abrogated the ability of Foxp3 to function as a repressor of IL-2 production (Fig. 1*D*). These results indicate that Foxp3 has the ability to inhibit cytokine gene expression in T cells, and the first 200 aa in the N terminus are critical for this inhibition.

The effect of Foxp3 on multiple cytokine expressions raised the possibility that Foxp3 might not function as a conventional transcriptional repressor that binds to the IL-2, IL-4, and IFN-γ promoters to suppress their activities. It is possible that Foxp3 is

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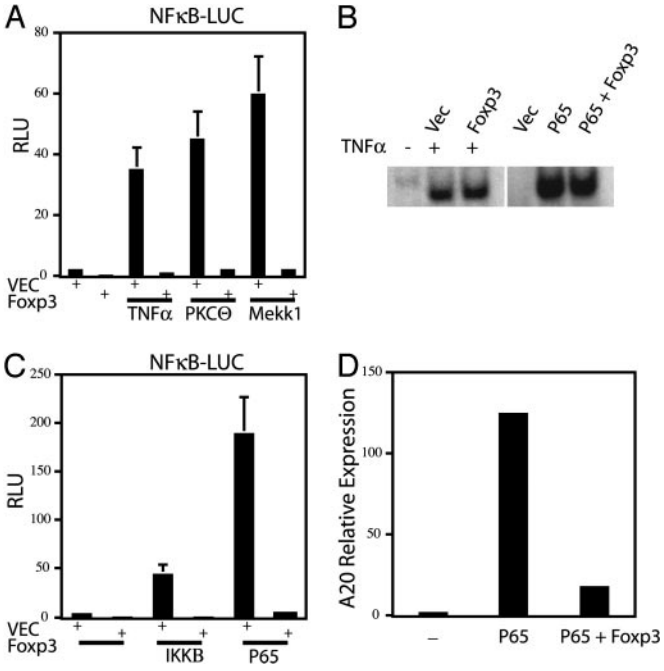

Fig. 2. Foxp3 inhibits NF-κB transcriptional activity and represses the endogenous expression of the NF-κB target gene, A20. (*A* and *C*) 293T cells were transfected with the indicated plasmids along with NF-κB luciferase reporter and Tk-*Renilla* reporter as an internal control. Twenty-four hours later (and an additional 6 h when cells were stimulated with TNF-α), cells were analyzed by means of the dual luciferase assay, with relative activity determined after normalization for *Renilla*. (*B*) 293T cells were transfected with Foxp3 and 48 h later stimulated for 1 h with TNF-α or transfected with P65 along with Foxp3, and NF-κB DNA-binding activity was assessed by EMSA. (*D*) 293T cells were transfected with the indicated plasmids, and, 48 h posttransfection, endogenous A20 expression was determined by real-time PCR.

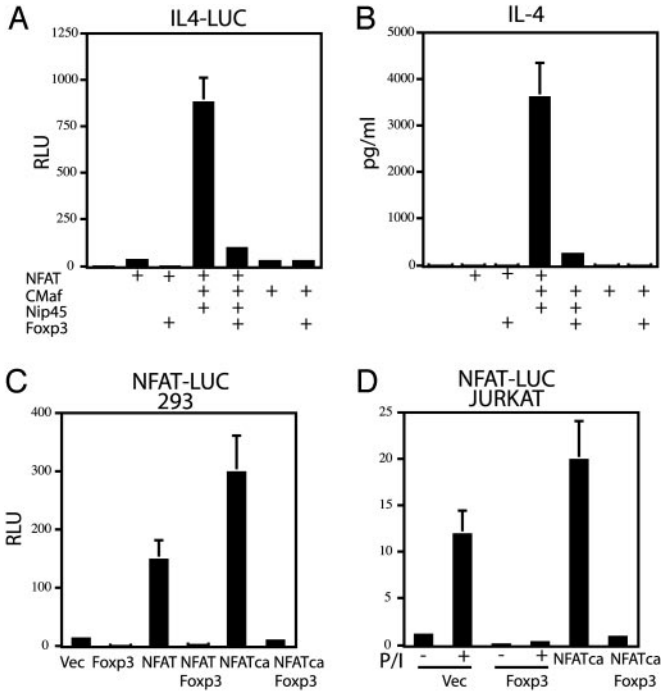

Fig. 3. Foxp3 inhibits NFAT transcriptional activity and represses the production of endogenous IL-4. (*A*) M12 cells were transiently transfected with the indicated expression plasmids along with the IL-4 luciferase reporter. After transfection, half of the transfected cells were cultured for 24 h and assayed for luciferase activity. (*B*) Cells from the other half were cultured for 72 h, and the supernatants were assayed for IL-4. Results are representative of three independent experiments. 293T cells (*C*) or Jurkat cells (*D*) were transfected with NFATp, a constitutively active NFATp (NFAT-CA), or a pcDNA3 vector control (Vec.) along with or without Foxp3 as indicated. In all cases, the NFAT luciferase reporter and the TK-*Renilla* reporter (as an internal control) were used (see *Materials and Methods*). Twenty-four hours later, cells were analyzed by means of the dual luciferase assay, with relative activity determined after normalization with *Renilla*. (*D*) Jurkat cells were transiently transfected as indicated above and analyzed by means of the dual luciferase assay after 6 h of stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (P/I).

able to suppress a broad range of cytokines by actually blocking the activity of transcriptional activators that are required for the transactivation of multiple cytokine genes (such as NFAT and NF-κB).

Foxp3 Is an Inhibitor of NF-κB Activation. Interestingly, when overexpressed in 293T cells, Foxp3 inhibited the basal level of NF-κB activation and also NF-κB activation mediated by PKC-θ, IκB kinase β (IKKβ), or TNF-α stimulation (Fig. 2*A*). To test whether NF-κB nuclear translocation was altered in the presence of Foxp3, we measured the NF-κB DNA binding activity by EMSA upon activation, and the translocation of NF-κB from the cytosol to the nucleus by immunohistochemistry. We did not observe any difference in DNA-binding activity (Fig. 2*B*) in the presence of Foxp3, nor in the NF-κB nuclear translocation (data not shown). These results suggest that Foxp3 might mediate its effect by directly blocking NF-κB transcriptional activity. To test this possibility, we overexpressed NF-κB (P65) in 293T cells and measured NF-κB activation in the presence of Foxp3. As presented in Fig. 2*C*, Foxp3 inhibits P65-mediated activation, thereby showing that NF-κB itself is a primary target of Foxp3. Foxp3 affects NF-κB transcriptional ability and not its DNA-binding activity (see Fig. 2*B*). Before addressing the molecular mechanism of Foxp3-mediated NF-κB repression, we wished to study whether endogenous NF-κB target genes were affected by Foxp3. The TNF-α-inducible antiapoptotic gene A20 is a well known NF-κB target gene (24). We tested whether the endogenous A20 mRNA level was affected by Foxp3 expression. 293T cells were transiently transfected by Foxp3, and extracted RNA was used to monitor the level of the A20 mRNA by real-time PCR. As presented in Fig. 2*D*, the overexpression of NF-κB (P65) alone is sufficient to turn on A20 expression. However, A20 expression induced by P65 is greatly repressed in the presence of Foxp3. Similar results were obtained with another NF-κB target gene, $cIAP_2$ (data not shown).

NFAT-Mediated Gene Expression Is Blocked by Foxp3. We have shown that Foxp3-expressing $CD4^+$ T cells produce fewer cytokines, including IL-4, upon TCR activation. In T cells, IL-4 expression is mainly controlled by the transcription factors NFAT and c-Maf. We wished to examine the effect of Foxp3 on endogenous IL-4 gene expression. Although M12 cells do not normally express IL-4, it has been shown that coexpression of the transcription factors c-Maf, NFAT, preexisting (NFATp), and the NFAT-interacting protein NIP45 leads to the induction of endogenous IL-4 production (25). Consistent with previous reports, we observed that c-Maf alone induced IL-4 promoter activity in M12 cells over the baseline, but its activity did not change in the presence of Foxp3 (Fig. 3*A*). Similarly, NFAT increased IL-4 promoter activity, but, in contrast to c-Maf, NFAT was unable to function in the presence of Foxp3 (Fig. 3*A*). Interestingly, overexpression of NFATp, c-Maf, and NIP45 led to the production of 3,000 pg/ml IL-4, and this IL-4 production was dramatically diminished (280 pg/ml) in the presence of Foxp3 (Fig. 3*B*). Foxp3 suppressed NFAT-dependent activation

when NFAT was overexpressed in 293T cells (Fig. 3*C*) and in Jurkat cells when the endogenous NFAT activity was induced upon phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation (Fig. 3*D*). We also used NFAT-CA, a constitutively active version of NFATp. NFAT-CA bears alanine substitutions for 12 phosphorylated serines whose dephosphorylation is normally required for nuclear localization. NFAT-CA is constitutively nuclear under conditions where endogenous NFAT is fully localized to the cytoplasm (26). When overexpressed, NFAT-CA is more potent in driving NFAT-luciferase activity than the WT NFATp version. Foxp3 suppressed both NFAT-CA and NFATp activity in 293T and in Jurkat cells (Fig. 3 *C* and *D*). This result strongly suggests that Foxp3 does not suppress NFAT activity by interfering with its nuclear translocation. It has been suggested that the Foxp-binding site within the IL-2 promoter is juxtaposed to the NFAT-binding site, and that Foxp3 might prevent NFAT protein binding to its DNA site (19). However, we did not find any difference in NFAT DNA binding activity when Foxp3 was overexpressed (data not shown). Foxp3 may inhibit NFAT function by interfering with its ability to transactivate. Based on this data, one could speculate that Foxp3 inhibits both NFAT and NF-κB activity in a similar fashion. One possibility is that Foxp3 regulates the expression of specific coactivators essential for both NFAT and NF-κB activity. Another possibility is that Foxp3 acts as a specific corepressor that physically associates with NFAT and NF-κB and directly inhibits their transcriptional activity.

Foxp3 Physically Associates with the REL Proteins NFAT and NF-κB and Represses Their Ability to Transactivate. First, we asked whether Foxp3 could physically interact with NFAT and NF-κB. Expression vectors encoding Flag-tagged Rel A subunit P65 (NFκB) or GFP tagged-P65, along with a Myc-tagged version of Foxp3, were overexpressed in the 293T kidney epithelial cell line. Coimmunoprecipitation using a monoclonal anti-Myc antibody and Western blotting with anti-P65 specific antibody revealed that Foxp3 physically associates with the P65 (Fig. 4*A*). We also tested the association of endogenous, rather than overexpressed, P65 with ectopically expressed Foxp3. 293 T cells were transduced with an HA-tagged Foxp3 expressing retrovirus and stimulated with TNF-α. Coimmunoprecipitation using a monoclonal anti-HA antibody and Western blotting with anti-P65 specific antibody revealed that Foxp3 also physically associates with endogenous P65 (Fig. 4*B*).

We also studied whether Foxp3 could physically interact with NFAT. Expression vectors encoding HA-tagged NFAT-CA or the HA-tagged nuclear kinase, RSK2 (used as a negative control) were overexpressed in 293T cells along with a Myc-tagged Foxp3. Coimmunoprecipitation using a monoclonal anti-Myc antibody and Western blotting with anti-HA-specific antibody revealed that Foxp3 physically associates with NFAT but not with RSK2 (Fig. 4*C*). Altogether, these results clearly show that Foxp3 physically associates with the REL domain contained transcription factors NFAT and NF-κB.

Often, transcription factors function within a large protein complex and require cooperative protein interactions to bind DNA with greater affinity. Even though we did not see a difference in NFAT and NF-κB DNA binding activity by EMSA in the presence of Foxp3, it is still possible that Foxp3 interferes with NFAT and NF-κB DNA binding activities on native promoters. To formally test these hypotheses, we fused the Gal4 DNA binding domain to NFAT and to p65, and cotransfected these chimeric cDNAs with a Gal4 binding site-luciferase reporter construct into 293T cells. We also used Gal4/ELK1 and Gal4/KRC chimeric constructs as specificity controls (21). All of the chimeric proteins potently transactivated the reporter construct, but Foxp3 inhibited the activity of only Gal4/NFAT and Gal4/p65, demonstrating that Foxp3 acts as a specific transcriptional corepressor for NFAT and NF-κB (Fig. 4*D*).

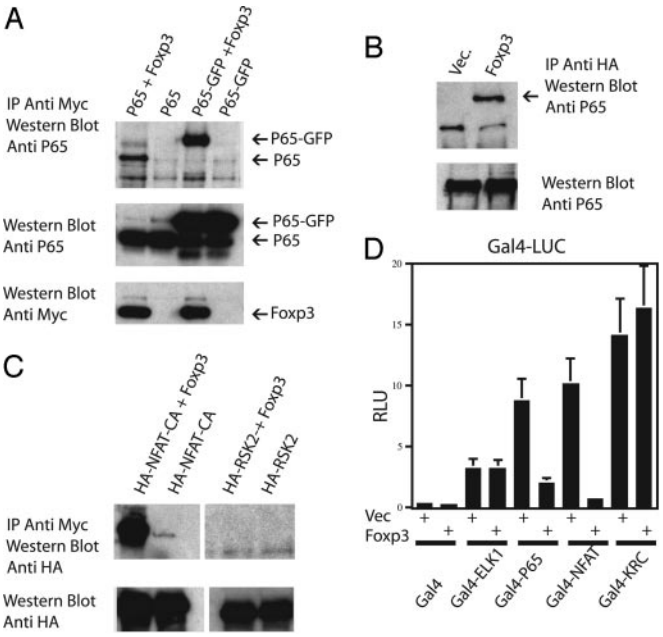

Fig. 4. Physical association between Foxp3 and the REL proteins NFATp and NF-κB and repression of their transactivation domain. (*A*) 293T cells were transiently transfected with P65, or with P65-GFP, along with Myc-Foxp3 or control vector and immunoprecipitated by using an anti-Myc monoclonal antibody. Proteins were run on SDS/PAGE and immunoblotted with a rabbit anti-P65 polyclonal antibody to detect the immunoprecipitates, and with anti-p65 or anti-Myc antibodies for the lysates. (*B*) 293T cells were transduced with RV control or with RV-HA-Foxp3, stimulated with TNF-α for 1 h, and immunoprecipitated by using an anti-HA monoclonal antibody and immunoblotted with a rabbit anti-P65 polyclonal antibody to detect the immunoprecipitate and the lysates. (*C*) 293T cells were transiently transfected with HA-tagged NFAT-CA or RSK2 along with Myc-Foxp3 or control vector and immunoprecipitated by using an anti-Myc monoclonal antibody. The immunoblotting was performed with a rabbit anti-HA polyclonal antibody to detect the immunoprecipitates and the lysates. (*D*) 293T cells were transfected with a Gal4 luciferase reporter along with a Gal4 DNA-binding domain, Gal4-P65, Gal4-NFAT, Gal4-KRC, or Gal4-ELK1 with or without Foxp3. Twenty-four hours later, cells were harvested and analyzed for luciferase activity as described in *Materials and Methods*.

Increase of NFAT and NF-κB Transcriptional Activity in Foxp3-Deficient T Cells. We have so far shown that Foxp3 is a specific corepressor for NFAT and NF-κB. However, this conclusion is based on studies performed under nonphysiological conditions. It was thus critical to investigate the role of Foxp3 under more physiological conditions by using primary T cells instead of cell lines. For this purpose, we used T cells derived from Foxp3-deficient mice. T cells derived from WT littermate control or from the Foxp3-deficient scurfy mice were purified, allowed to rest for 24 h in the presence of IL-2, and transfected with NFAT or NF-κB luciferase reporter. Upon anti-CD3 and anti-CD28 activation, Foxp3-deficient T cells displayed a 20-fold increase in NFAT and a 30-fold increase in NFκB activities over the WT-derived T cells (Fig. 5 *A* and *B*).

Furthermore, when we reintroduced Foxp3 into scurfy-derived T cells, we were able to diminish the NFAT and NF-κB transcriptional activities closer to the activities found in WT T cells. Foxp3 suppression of NFAT and NF-κB was specific because Foxp3 did not affect the *Renilla* activity driven by the Thymidine kinase promoter used in our assay to normalize transfection efficiency. Moreover, Foxp3-mediated NFAT and

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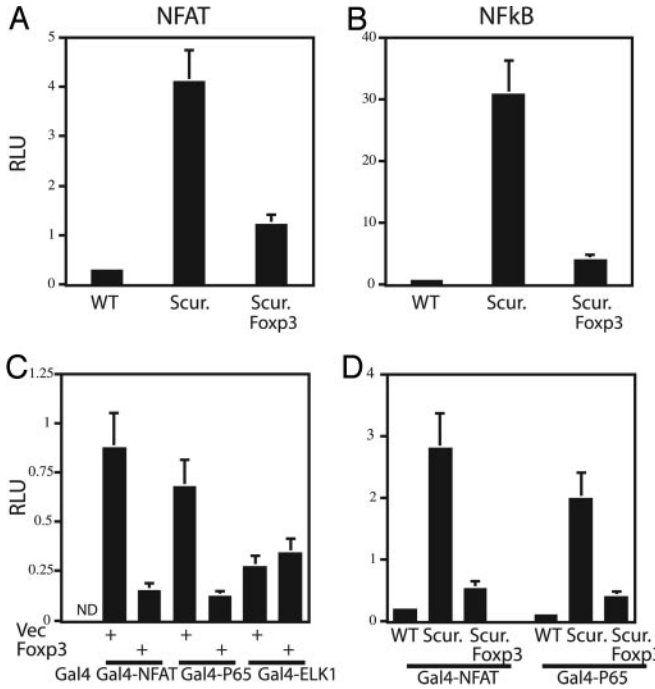

Fig. 5. The absence of a functional Foxp3 leads to NF-κB and NFAT hyperactivation. NFAT (*A*) and NF-κB (*B*) luciferase activities were assessed in primary CD4$^+$ T cells derived from WT scurfy mice (Scurf.) or scurfy T cells transfected with Foxp3 and activated with anti-CD3 and anti-CD28 for 24 h. Primary T cells derived from WT (*C*) or from scurfy mice (*D*) were transfected with a Gal4 luciferase reporter along with a Gal4 DNA-binding domain, Gal4-P65, Gal4-NFAT, or Gal4-ELK1 with or without Foxp3 and activated with anti-CD3 and anti-CD28. Twenty-four hours later, cells were harvested and analyzed for luciferase activity as described in *Materials and Methods*.

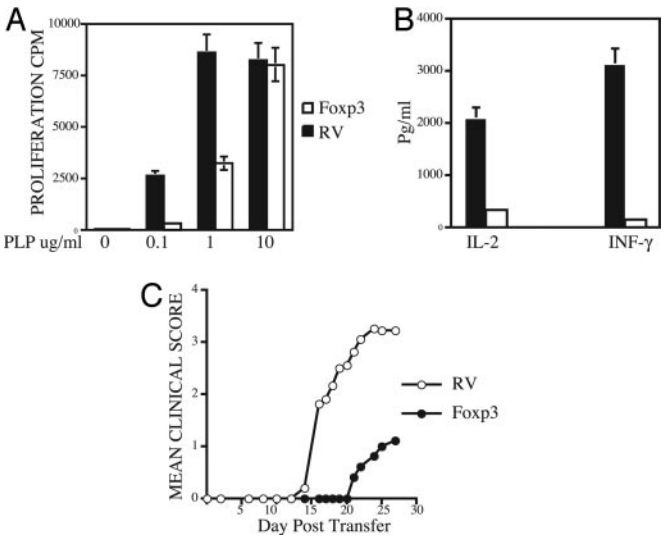

Fig. 6. Effect of Foxp3 on the *in vivo* effector function of CD4$^+$ T cells. (*A*) CD4$^+$ T cells from PLP-specific TCR transgenic mice were purified, activated with PLP139-151, and infected with RV or RV-Foxp3 retroviruses. CD4$^+$ GFP$^+$ from RV- or RV-Foxp3-infected cells were then sorted and tested for their proliferative response toward PLP peptide by ^{3}H incorporation. The data represent the proliferative response ± SE. (*B*) The figure represents the amount of IL-2 and IFN-γ secreted in the culture supernatant of these activated cells as measured by ELISA. (*C*) GFP$^+$-sorted PLP-specific T cells (5×10^6) infected with RV or RV-Foxp3 were injected into RAG-deficient mice. The progression of EAE was followed over time and is presented as the mean clinical disease among each group.

NF-κB repression in primary T cells was attributed to the ability of Foxp3 to specifically suppress NFAT and NF-κB transactivation domains, because it did not affect ELK transactivation (Fig. 5*C*). More importantly, scurfy-derived T cells displayed higher NFAT and NF-κB transactivation activity compared with the WT-derived T cells. This abnormal elevated transactivation activity was dramatically reduced when Foxp3 was introduced into these T cells (Fig. 5*D*). These results clearly show that, under physiological conditions, Foxp3 indeed acts as a specific transcription corepressor for Rel-domain contained transcription factors NF-κB and NFAT. Foxp3 is a potent immunosuppressive gene that exerts its function by means of the suppression of NF-κB and NFAT, which are essential for T cell effector function.

Foxp3 Expression Inhibits Pathogenic Activity of Autoreactive T Cells. We investigated whether Foxp3-transduced T cells could modulate the effector function of pathogenic autoreactive T cells. We purified CD4$^+$ T cells from the autoantigen PLP-specific TCR transgenic mice (5B6), stimulated them with PLP139-151 peptide and syngenic antigen-presenting cells, and infected with either RV or RV-Foxp3 retroviruses. CD4$^+$ GFP$^+$ from RV- or RV-Foxp3-infected cells were then sorted and tested for their proliferative response and their effector cytokine production toward PLP peptide. Although the control RV-infected cells still significantly proliferated to the PLP139-151 peptide, there was a significant decrease in the proliferative response of RV-Foxp3-infected cells (Fig. 6*A*). Furthermore, whereas the RV-transduced PLP-specific T cells produced a large amount of IL-2 and INF-γ, the Foxp3-transduced T cells produced significantly much fewer effector cytokines (Fig. 6*B*). Next, we investigated whether Foxp3-transduced T cells could modulate the effector function of pathogenic autoreactive T cells *in vivo*. Infected 5B6 T cells, with either RV or RV-Foxp3 retroviruses, were transferred into syngenic RAG-deficient mice. At day 14 after the transfer, mice that were transferred with RV-infected cells started to develop a severe progressive paralysis (Fig. 6*C*). By day 20, although all mice in the RV-transferred group developed EAE with scores of 2 and above, none of the RAG-deficient mice that were transferred with RV-Foxp3 had developed any sign of disease. Eventually, some of the mice transferred with RV-Foxp3 cells developed EAE; however, the clinical signs were much milder than those observed in the groups transferred with RV control transduced T cells. These results demonstrated that the expression of Foxp3 in CD4$^+$ T cells significantly limits the encephalitogenic potential of T cells and interferes with their effector functions *in vivo*.

Discussion

We have shown that, among the Foxp transcriptional factor family, which includes Foxp1, Foxp2, and Foxp3, only Foxp3 has the ability to inhibit IL-2, IL-4, and INF-γ production in primary T helper cells. Considering that all of the Foxp family proteins carry a similar DNA-binding domain, and that Foxp1 and Foxp3 are able to bind and suppress the IL-2 promoter reporter activity, we expected that they would share some overlapping functions in T cells. However, they do not, because Foxp3 is the only Foxp member that has the ability to regulate cytokine gene expression in T cells. One possible explanation is that Foxp3 mediates its effect by repressing the activity of transcriptional activators necessary for cytokine gene expression. Indeed, we found that Foxp3 functionally and physically interacts with the NFAT transcription factor, such that NFAT was unable to turn on IL-4 expression in the presence of Foxp3. Additionally, we found that Foxp3 physically associates with NF-κB and blocks its ability to induce the expression of the NF-κB-dependent gene, A20. More

importantly we have shown that T cells derived from scurfy mice, which lack a functional Foxp3 protein, have a dramatic increase in endogenous NFAT and NF-κB transcriptional activities compared with that of WT T cells. Furthermore, if we introduce Foxp3 back into these scurfy-derived T cells, Foxp3 is able to suppress NFAT and NF-κB transcriptional activities. This result clearly shows that, under physiological conditions, Foxp3 acts as a specific transcription corepressor for two key transcription factors (NF-κB and NFAT) essential for the expression of many cytokine genes.

The scurfy mice exhibit a severe lympho-proliferative disorder and display a progressive lymphocytic infiltration of the lymph nodes, spleen, and skin, resulting in gross morphologic symptoms that include splenomegaly, greatly enlarged lymph nodes, runting and death at 3 weeks of age. The disease has been shown to be primarily mediated by $CD4^+$ T lymphocytes, suggesting that Foxp3 plays an important role in regulating T cell function. This T cell defect is primarily manifested as a generalized overproduction of cytokines (12, 14). Our finding that Foxp3-deficient T cells have an abnormally elevated NFAT and NF-κB activity upon anti-CD3 and anti-CD28 stimulation may explain the excessive production of these cytokines, which correlates well with the range of pathologic changes observed in the mutant mice. For instance, an excessive NF-κB activation leads to spontaneous inflammation, cachexia, and premature death as seen in the A20, Foxo3, and Foxj1-deficient mice. At the steady state in T cells, NF-κB is trapped in the cytosol by the IκB proteins and released from them upon activation. Interestingly, Foxo3 and Foxj1 control the expression of IκBβ and IκBε, and therefore indirectly repress NF-κB activation by preventing its translocation to the nucleus (27, 28). Foxp3 represses NF-κB by a different mechanism, however; Foxp3 directly interacts with NF-κB and represses its ability to transactivate. We think that Foxp3 is probably recruited to the promoters of cytokine genes by NF-κB and NFAT. Because Foxp3 is intrinsically a very potent transcriptional repressor, its recruitment to the promoter would turn off any transcriptional activity.

Recently, it was shown that Foxp3 is directly involved in the generation of the $CD4^+CD25^+$ T regulatory cells, and that the fatal lympho-proliferation observed in the Foxp3-deficient mice could result from a deficiency in these $CD4^+CD25^+$ Tregs. However, the phenotype of animals lacking Foxp3 is far more dramatic than the phenotype of most of the experimental functions ascribed to the $CD4^+CD25^+$ Tregs *in vivo* (15–17). Although scurfy mice succumb to their autoimmune disease by 3 weeks of age, the absence of Tregs results in autoimmunity and not a rapid lethal phenotype (29). Furthermore, whereas most of the $CD4^+CD25^+$ Tregs are generated in the thymus, overexpression of Foxp3 in the thymus is unable to prevent disease in mice lacking a functional Foxp3 gene. Thus, Foxp3 has an equally important function in peripheral $CD4^+$ T cells (14), and Foxp3-deficient T cells are hyperresponsive to low amounts of TCR stimulation. This decreased requirement for costimulation through CD28 indicates that these T cells have an intrinsic defect and have a low activation threshold (30). We have also shown that PLP-specific autoreactive T cells transduced by Foxp3 are less capable of mediating EAE. This result suggests that, besides generating T regulatory cells (which may be a thymic-driven event) Foxp3, by repressing NFAT and NF-κB activity, has another important function on mature T cells, in that it inhibits proliferation and effector function of peripheral T cells.

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